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Cadmium Uptake and Metabolism in Cultured Cells

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Cultured cells have been made resistant to otherwise lethal concentrations of the toxic Cd ion, probably by induction of metallothionein (MT) synthesis and binding of Cd to the MT. One human epithelial cell line (HE) and two enzyme-deficient mutants of mouse fibroblasts (L-cells) (Cl 1D and A9) and their Cd-resistant substrains with a high content of MT, have been used to study cellular Cd uptake and metabolism. For cell survival of "wild type" cells, the critical level of intracellular Cd is determined to be around 6 nmole Cd/mg cell protein. Resistant cells can tolerate Cd levels several times above this concentration, if the major part of Cd is bound to MT. The technique of perturbed angular correlation spectroscopy (PAC) has been applied to living Cd-resistant cells. It was shown that > 66% of Cd in the resistant strains was bound to MT, and that MT is apparently freely suspended in the cell cytoplasm. Chelating agents differ in toxicity and Cd-releasing effect on the cells, but apparently remove the non-MT-bound Cd pool. After various periods of Cd omission, either *in vitro* or *in vivo*, growing the cells as tumors in athymic nude mice, the stability of Cd resistance in these cells seems to be dependent on the capacity of cells for *de novo* synthesis of MT shortly after re-exposure to the metal.

Introduction

Cultured cells can be adapted to high concentrations of the toxic cadmium ion, apparently by induction of synthesis of the Cd-binding protein metallothionein (MT) (*1,2*). This is one of several indications that MT probably plays a role in Cd detoxification (*3*). We have recently obtained results which indicate that MT may protect cells from toxic effects of other metals (*4*) and metal-containing drugs (*5*), as well as certain alkylating agents (*6*) and ionizing radiation (*7*). In view of the various possible biological functions of MT, we also have found it of interest, in our laboratory, to evaluate the importance of MT as a protective factor against cadmium toxicity. It is the purpose of this paper to present these studies, which are performed mainly as *in vitro* studies on cultured cells.

Cd Resistance and MT Levels in Cultured Cells

Rugstad and Norseth have described the development of Cd-resistant substrains of a human epithelial cell line (*1*) and two enzyme-deficient mutants of mouse fibroblasts (L-cells) (*2*). The human cells were derived from normal skin, and their epithelial origin was confirmed by phase contrast microscopy and electron microscopy (*8*). Their human origin was demonstrated by isoenzyme studies and by typing surface antigens by a microcytotoxicity assay (*8*). The murine cells were two different fibroblast lines: one is designated clone 1D and is deficient in thymidine kinase; the other is designated A9 and is deficient in hypoxanthine-guanine phosphoribosyl transferase. Substrains resistant to otherwise letal doses of $CdCl_2$, namely 100 μmole/L of culture medium, were developed from all three parent cell lines. This was performed by exposing cells to stepwise increased concentrations of $CdCl_2$. The lysates of the Cd-resistant cells, but not those from nonresistant cells, contained large amounts of low molecular weight cadmium binding proteins. These proteins were determined to be metallothionein (MT) by amino acid analysis (*5*). There is good evidence that the resistance in these cells is due to induced synthesis of MT and binding of Cd to MT, thereby protecting the cells from the otherwise lethal effect of such high total intracellular concentrations of cadmium.

We also suggest that the pattern of develop-

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ment of Cd resistance in these cells might be compatible with an amplification of the MT gene.

These cells have been used for further studies on Cd metabolism and experiments to reveal functions of the MT. Culture methods and experimental conditions are detailed elsewhere (9–12).

Results

Cd Content and Egress from Cells with and without MT

One of the most common mechanisms of resistance to drugs or other toxic substances is the cells' ability to reduce the intracellular concentration of the substance concerned. This may be caused either by reduced uptake or enhanced efflux from cells. Bakka and Rugstad (*9*) have investigated the concentration of Cd in the three Cd-resistant cell strains, compared to their parent cell lines by exposing cells to [^{109}Cd]-$CdCl_2$, to reveal whether or not decreased amounts of intracellular Cd might contribute to the Cd resistance in these cells. The results were principally the same for all three Cd-resistant substrains used. Figure 1 shows the results from experiments with the

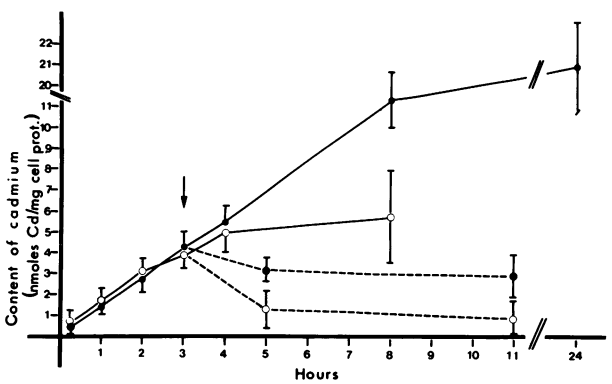

FIGURE 1. Content of Cd (○) in Cl 1D and (●) Cl $1D_{100}$ cells after incubation in culture medium containing 100 μmole $CdCl_2$/L. C1 $1D_{100}$ cells were previously adapted to the same concentration of $CdCl_2$ and contained high amounts of metallothionein. (----) Release of Cd from cells growing in medium without Cd, following 3 hr preincubation with 100 μmole Cd/L. The arrow indicates start of release experiment. Each point represents the mean ± SD from 12 cultures. From Bakka and Rugstad (*9*) with permission from the journal.

clone $1D_{100}$ cells. There was no significant difference in Cd content between the Cd-resistant strains and the nonresistant parent cell lines during the first 4 hr of incubation. After that point, the resistant strain proliferated with a concentration of cellular Cd much higher than that which after about 8 hr incubation injured the nonresistant cells to a degree not compatible with further growth. The critical level of intracellular Cd which cannot be exceeded without lethal consequences on nonresistant cells seems to be 6–8 nmole/mg cell protein, whereas the resistant cells contained 20–30 nmole/mg after 24 hr growth with $CdCl_2$. At that point the resistant cells still had a normal appearance as judged by phase contrast microscopy and were capable of apparently normal growth. After omission of Cd from the medium, the egress of Cd during the first 2 hr occurred more rapidly and to a greater extent from the nonresistant strain than from the resistant strain. After about 2 hr, the Cd egress was the same from both cell strains.

Recent experiments in which the Cd-resistant MT-containing substrain of human epithelial cells was exposed to gold (III) chloride show that these cells in addition are resistant to concentrations of gold chloride which are lethal to the parent cell line, and that the Cd-resistant cell strain is capable of growing with a higher concentration of intracellular gold than that which kills the nonresistant cell strain (*4*).

Cd-Depleting Effect of Chelating Agents

One of the main problems in treatment of Cd poisoning in humans is the relative inefficiently chelation therapy. One probable reason is that most of the Cd is tightly bound to intracellular proteins, in particular to MT. An efficient chelating agent has to meet several requirements: the chelating agent must have acceptable toxic effects on cells; the complex of the chelating agent and the metal must have acceptable toxic effects; the chelating agent must penetrate into the cells; the complex of the chelating agent and the metal must be released from cells; and the complex must be excreted from the body. Cultured cells with a high content of Cd and MT are considered to be a useful *in vitro* model for primary screening of different chelating agents regarding some of these requirements, especially those concerning potentially toxic effects of the substances themselves and their ability to increase the release of Cd from the cells. The term "release" is used to designate the overall transfer of metal from cells to the growth medium. Bakka et al. (*10*) studied the effect of six different metal chelating compounds on cell growth of the Cd-resistant, MT-containing substrain of the human epithelial cell line and also the effect of chelators on the efflux of

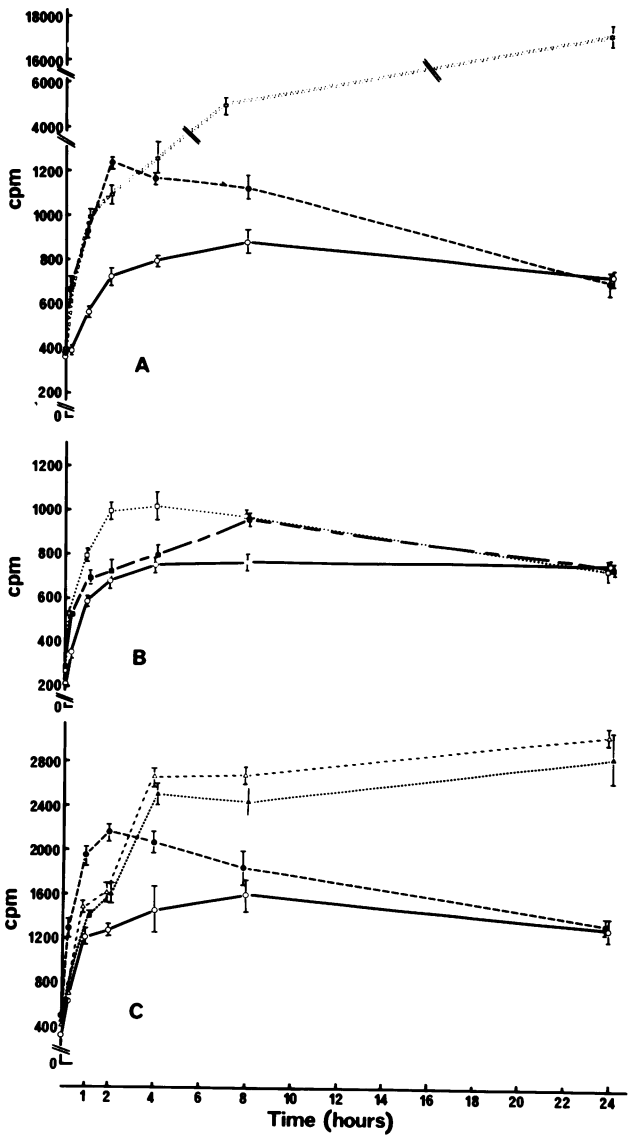

FIGURE 2. Release of ^{109}Cd into the culture medium from human epithelial cells (HE_{100}) previously adapted to 100 μmole $CdCl_2$/L culture medium, and containing high amounts of metallothionein during treatment with chelating agents at equimolar concentrations of 1 mmole/L: (*A*) control cells grown (○) without addition of chelating agent, (●) grown with 2, 3-dimercaptopropane-1-sulfonate (BAL-sulph.) and (*▽△) grown with 2, 3-dimercapto-1-propanol (BAL); (*B*) controls grown (○) without agent; (□) cells grown with D-penicillamine (PA) and (■) with *N*-acetyl-DL-penicillamine (NAPA); (*C*) controls (○) without agent; (●) cells grown with BAL-sulph., (△) with mercaptosuccinic acid and (▲) with meso-2,3-dimercaptosuccinic acid (DMSA). Ordinate gives the CPM per volume unit of the medium, abscissa indicates time from onset of chelator exposure. Each point gives the mean ± SD of 6 replicates. From Bakka et al. (*10*) with permission from the journal.

Cd from the cells. The release of ^{109}Cd from cells under influence of some of the chelating agents is shown in Figure 2. Of all the substances tested, 2,3-dimercaptopropane-1-sulfonate (BAL-sulph.) caused the most rapid release of Cd from cells during the initial 2 hr incubation, whereas 2,3-dimercapto-1-propanol (BAL) and mercaptosuccinic acid (MSA) were the most potent Cd-releasers during the first 24 hr of exposure. The toxicity studies determining cell growth during the influ-

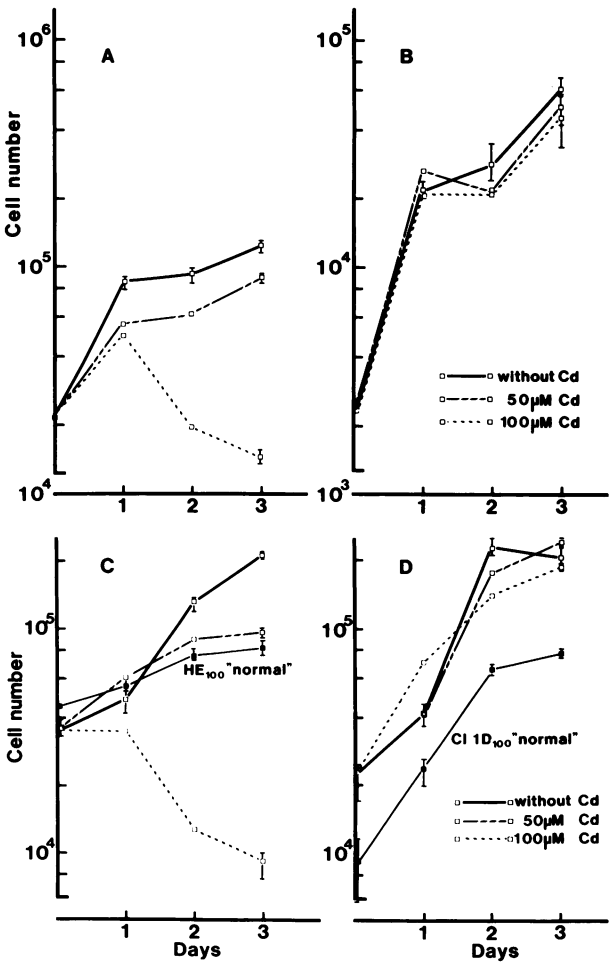

FIGURE 3. Cell growth during re-exposure to 50 or 100 μmole Cd/L culture medium, and in the absence of Cd, of previously Cd-adapted human epithelial cells (HE_{100}) (*A, C*) and mouse fibroblasts (Cl $1D_{100}$) (*B, D*), following omission of Cd for 1 week (upper panel) or 3 weeks (lower panel). Both cell strains had previously been adapted to 100 μmole of Cd/L. Growth rates of the cells grown continuously with 100 μmole Cd/L (designated "normal") are demonstrated in the lower panel. Ordinate: Cell number (logarithmic scale). Abscissa: Time from onset of Cd re-exposure. Each point is the median of six replicates; bars represent the 25th and 75th percentiles for the distribution of cell counts. From Endresen et al. (*11*) with permission from the journal.

ence of the chelating agents revealed that BAL was more toxic to the cells than the other agents, indicating that the egress of Cd from cells caused by that agent to some degree probably was a leakage of Cd from cells with injured plasma membrane.

Stability of Cd Resistance and MT Levels

The possible biological function of MT as a detoxifying substance for Cd called for investigations on stability of the Cd resistance and the MT levels in Cd-adapted cultured cells, both of human and murine origin. Endresen et al. (*11*) expressed the degree of stability of Cd resistance as variations in cell growth during re-exposure to Cd following a period of omission of Cd from the culture medium (Fig. 3). After a 3-week omission period, the resistance was the same in the murine cells, but decreased in the human cell strain to 50% of initial values. Elution profiles obtained after gel filtration of cytosols (Fig. 4) revealed that after 3 weeks there was no detectable MT left in any of the two strains. Both strains had the capacity of rapid *de novo* MT synthesis even after 5 weeks without Cd. This capacity was best preserved in the mouse fibroblast strain, in which 80% of the previous levels of MT (during continuous growth in Cd-containing medium) could be demonstrated after 24 hr re-exposure to maximum tolerable doses, whereas only 60% was attained in the human cell strain.

Discussion

There is a striking difference in Cd tolerance between cultured cells, either human or murine, previously being adapted to 100 μmole Cd/L culture medium and the parent cells not previously exposed to Cd. The critical level of intracellular Cd in the parent cells described is about 6 nmole/mg cell protein, whereas the Cd-adapted strains grow continuously at levels 4 to 7 times higher. In the resistant strains, 95% of the intracellular Cd is bound to MT, as determined by gel filtration elution profiles. Thus the level of Cd not bound to MT is well below the level which is lethal to the parent cells. It is suggested that the binding of Cd to MT also explains the slow release of Cd from these cells compared to the parent cells, when Cd is removed from the medium. Bakka et al. (*12*) also used another method to study the binding modes of Cd in cells, namely, the spectroscopic technique of perturbed angular correlation of γ-rays (PAC). As far as we know, this is the first

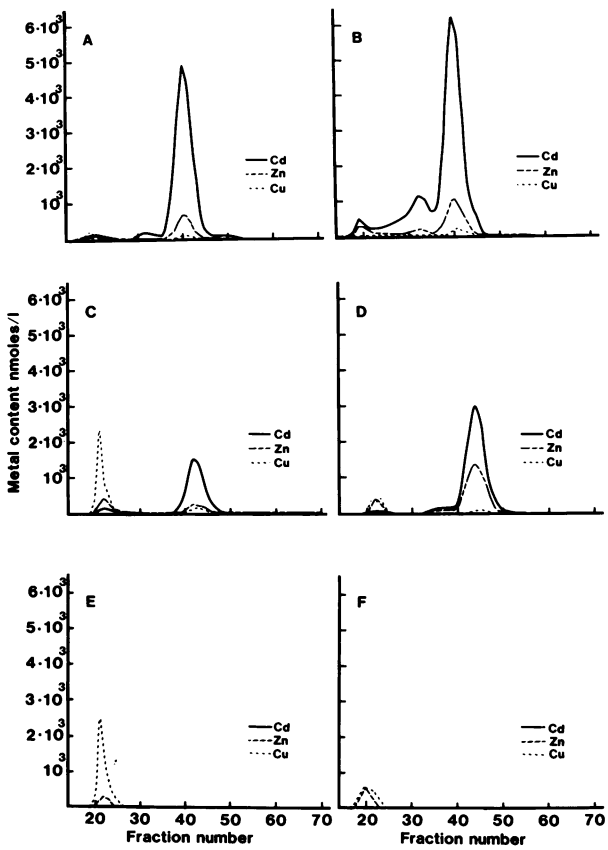

FIGURE 4. Elution profiles obtained after Sephadex G-75 chromatography of cytosols from previously Cd-adapted substrains of human epithelial cells (HE_{100}) (*A, C, E*), and mouse fibroblasts (Cl $1D_{100}$) (*B, D, F*), both previously been grown with 100 μmole Cd/L culture medium. The upper panels (*A, B*) demonstrate the "normal" Cd-adapted state of the cell strains, the lower panels (*E, F*) show the elution profiles for the cells after being cultured for 3 weeks without Cd-supplement, and the panels in the middle (*C, D*) the profiles after 5 weeks without Cd and subsequent re-exposure for 24 hr to maximal tolerable doses, that is 50 μmole Cd/L for the HE_{100} cells and 100 μmole Cd/L for the $1D_{100}$ cells. All data are expressed as metal concentration in each fraction and are normalized to 1 mg of cytosolic protein applied to the column. The peak eluting with fractions 20–25 represents the high molecular weight proteins (MW>75,000); the peak eluting with fractions 35–45 contains the metallothioneins (MW≈10,000). From Endresen et al. (*11*) with permission from the journal.

time PAC has been used for this purpose (*13*) in living cultured cells. Those studies confirm the conclusion that there are two different types of Cd binding sites within cells. MT constitutes the main one, binding >60% of the Cd. Additional important information is that this MT apparently is freely suspended in the cytoplasm of the cells. Thus, there is good evidence that MT is one main protecting factor against the toxic Cd ion in these

cells and that mechanisms leading to decreased concentration of Cd in the cells does not seem to be at work. This is in agreement with the suggested role of MT regarding resistance against gold (III) chloride. The fact that gold binds to the pre-existent Cd-induced MT in the Cd-resistant human epithelial cell strain probably protects cells from the otherwise lethal gold concentration (*4*).

Also the study on the effect and toxicity of chelating agents led to the assumption that Cd exists in at least two pools within the cells. One rather small part of the Cd is relatively loosely bound and can easily be removed within a few hours by a chelating agent. The other is tightly bound within cells and is removed slowly. It seems plausible to suggest that the small, loosely bound fraction represents a more toxic threat to the cells than does the major pool, which probably comprises the MT-bound Cd. An efficient chelating agent should preferably be able to release Cd from both pools. This capacity will be dependent on the relative affinities of Cd to the chelator versus the MT. None of the compounds tested appeared to have the capacity of releasing more than minor amounts of Cd bound to MT. It appears reasonable to test new chelators in systems including MT, and the *in vitro* system described by Bakka et al. (*10*) seems to be a promising system for primary screening of effect and toxicity of such agents. The high toxicity of BAL has also been demonstrated by others in studies on mice (*14*). The superior releasing effect of MSA *in vitro* calls for further investigation *in vivo*.

The demonstrated stability of the Cd-resistance in previously Cd-treated cell strains of both human and murine origin might be due to their capacity of immediate *de novo* MT synthesis when re-exposed to the Cd ion. This has also been demonstrated by *in vivo* studies, in which the murine cells were grown as tumors in athymic nude mice, that is, in the absence of Cd, and then re-exposed them to Cd after plating the tumor cells either in tissue culture flasks or in soft agar (*11*). This mechanism of preserving the acquired Cd resistance is compatible with the evidence presented by Hildebrand et al. (*15*) regarding Cd resistance in cultured CHO cells. In terms of genetics, the explanation for the difference in capacity for *de novo* MT synthesis between the murine and human cell strain is not yet known. One might speculate that differences in degree of amplification of the MT gene account for this difference. It is reported (*16*) that gene amplification occurs in Cd-resistant Friend leukemia cells, when compared to their parent nonresistant cells.

Conclusions

Studies on Cd uptake and metabolism in cultured murine and human cell strains with and without Cd-induced MT have revealed the following.

(1) The amount of non-MT-bound Cd seems decisive for Cd toxicity in cells. The critical level seems to be about 6 nmole/mg cell protein in three different cell lines. The resistant cells can tolerate total amounts of intracellular Cd which are several times higher, if the major part is bound to MT, and the pool of non-MT-bound Cd does not exceed this critical level.

(2) The PAC technique is well suited to study binding modes of Cd in living cells and indicates that the cellular Cd is mainly bound to MT and that Cd-containing MT molecules are freely suspended in the cell cytoplasm.

(3) An *in vitro* model of cultured cells containing MT seems to be a promising test system for primary screening of effect and toxicity of new chelators. Chelating agents apparently remove the non-MT-bound Cd pool in cells. MSA seems more efficient and less toxic than BAL.

(4) The stability of Cd resistance varies between human epithelial cells and mouse fibroblasts in culture. The evidence presented is in agreement with the concept that resistance to the potent MT-inducing metal Cd is stable due to the capacity of cells for *de novo* synthesis of MT shortly after re-exposure to the metal, both *in vitro* and *in vivo*.

REFERENCES

1. Rugstad, H. E., and Norseth, T. Cadmium resistance and content of cadmium-binding protein in cultured human cells. Nature 257: 136–137 (1975).
2. Rugstad, H. E., and Norseth, T. Cadmium resistance and content of cadmium-binding protein in two enzyme deficient mutants of mouse fibroblasts (L-cells). Biochem. Pharmacol. 27: 647–650 (1978).
3. Kägi, J. H. R., Kojima, Y., Kissling, M. M., and Lerch, K. Metallothionein: an exceptional metal thiolate protein. In: Sulfur in Biology (Ciba Foundation Symposium 72), Exerpta Medica, 1980, pp. 223–237.
4. Glennås, A., Bakka, A., and Rugstad, H. E. Cultured human cells with a high content of metallothionein show resistance against gold-chloride. Scand. J. Rheumatol. in press.
5. Bakka, A., Endresen, L., Johnsen, A. B. S., Edminson, P. D. and Rugstad, H. E. Resistance against *cis*-dichlorodiammineplatinum in cultured cells with a high content of metallothionein. Toxicol. Appl. Pharmacol. 61: 215–226 (1981).
6. Endresen, L., Bakka, A., and Rugstad, H. E. Increased resistance to chlorambucil in cultured cells with a high concentration of cytoplasmic metallothionein. Cancer Res. 43: 2918–2926 (1983).

7. Bakka, A., Johnsen, A. S., Endresen, L., and Rugstad, H. E. Radioresistance in cells with high content of metallothionein. Experientia 38: 381–383 (1982).
8. Ree, K., Johnsen, A. S., Rugstad, H. E., Bakka, A., and Hovig, T. Characterization of a human epithelial cell line with special reference to its ultrastructure. Acta Pathol. Microbiol. Scand. A89: 73–80 (1981).
9. Bakka, A., and Rugstad, H. E. Uptake and egress of cadmium in cultures of cadmium-resistant and the corresponding "wild-type" cells. Acta Pharmacol. Toxicol. 48: 81–86 (1981).
10. Bakka, A., Aaseth, J., and Rugstad, H. E. Influence of certain chelating agents on egress of cadmium from cultured epithelial cells containing high amounts of metallothionein: a screening of Cd-releasing and toxic effects. Acta Pharmacol. Toxicol. 49: 432–437 (1981).
11. Endresen, L., Bakka, A., Glennås, A., Tveit, K. M., and Rugstad, H. E. Stability of cadmium resistance and metallothionein levels *in vitro* and *in vivo*. Toxicol. Appl. Pharmacol. 67: 274–283 (1983).
12. Bakka, A., Eriksen, D. Ø, Rugstad, H. E., and Bauer, R. Identification of cadmium binding sites within living human cells by perturbed angular correlation spectroscopy. Febs Letters 139: 57–60 (1982).
13. Adloff, J. P. Application to chemistry of electric quadrupole perturbation of γ - γ angular correlations. Radiochim. Acta 25: 57–74 (1978).
14. Zwirblis, P., and Ellin, R. I. Acute toxicity of pure dimercaprol and trimercaptopropane. Toxicol. Appl. Pharmacol. 36: 297–299 (1976).
15. Hildebrand, C. E., Griffith, J. K., Tobey, R. A., Walters, R. A., and Enger, M. D. Molecular mechanisms of cadmium detoxification in cadmium-resistant cultured cells: role of metallothionein and other inducible factors. Devel. Toxicol. Environ. Sci. 9: 279–303 (1982).
16. Beach, L. R., and Palmiter, R. D. Amplification of the metallothionein-I gene in cadmium resistant mouse cells. Proc. Natl. Acad. Sci. (U.S.) 78: 2110–2114 (1981).